2001

The role of matrix metalloproteinase-2 in glioma invasion

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The Role of Matrix Metalloproteinase-2 in Glioma Invasion

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Masters of Science in Biology
University of Richmond, 2001
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Abstract

Overexpression of matrix metalloproteinase 2, MMP-2, is correlated with high-grade gliomas *in vivo*. The purpose of this study was to investigate whether MMP-2 overexpression in glioma cell lines leads to increased invasion of glioma cells *in vitro*. The entire MMP-2 gene was isolated from U87, a glioma cell line known to have high expression of wild-type MMP-2. This MMP-2 gene was inserted in a sense and antisense orientation into pCR3.1 vectors with a CMV promoter to ensure high levels of expression. Glioma cell lines were transfected with the sense and antisense constructs, as well as the pCR3.1 vector alone to serve as a control. Zymography and Western blot analysis of conditioned media taken from glioma cell lines U251, T98, and LNZ-308 transfected with sense MMP-2 revealed an overproduction of MMP-2 relative to controls. The invasiveness of these transfected cell lines was evaluated using an *in vitro* invasion assay. These assays revealed significantly more invasion by cells that were overproducing MMP-2 relative to cells containing either vector alone (control) or vector containing the antisense construct. These results support the hypothesis that glioma cell lines overexpressing MMP2 are more invasive *in vitro*.

I certify that I have read this thesis and find that, in scope and quality, it satisfies the requirements for the degree of Masters of Science.

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THE ROLE OF MATRIX METALLOPROTEINASE-2 IN GLIOMA INVASION

By

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B.A., LaSalle University, 1999

A Thesis

Submitted to the Graduate Faculty

of the University of Richmond

in Candidacy

for the Degree of

MASTER OF SCIENCE

in

Biology

June, 2001

Richmond, Virginia

Acknowledgement

I would like to thank Dr. Valerie Kish, my thesis advisor, for taking me into her laboratory and for all of her help and guidance over the past two years. I would also like to thank my thesis committee, Dr. Helen Fillmore and Dr. Krista Stenger for all of their support and direction. I would like to also express my deep gratitude to the members of the Broaddus/Fillmore laboratories, especially Dr. Helen Fillmore, Peggy Furqueron, and Binh Nguyen, for getting in on such exciting research, for their assistance in providing me with materials and protocols, as well as their continued guidance throughout this project. Additionally, I would like to thank the University of Richmond Graduate Research Committee for funding this research. Finally, I want to thank my parents for all of the their love and support.

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Introduction

Matrix metalloproteinase 2 (MMP-2) has recently been associated with glioma invasion. It is known to cleave components of the extracellular matrix including collagen type I, IV, and V, as well as, elastin, fibronectin, and laminin (Uhm et al., 1997). MMP-2 expression in several glioma cell lines has been manipulated through the use of sense and antisense human MMP-2 cDNA. The aim of this thesis research is to study the effects of changes in MMP-2 expression level on the invasiveness of gliomas.

Gliomas are brain tumors that begin in the glial cells, or supportive cells of the brain. Despite modern advances in surgical techniques, neuroimaging, chemotherapeutic agents, and radiation strategies, glial tumors are basically incurable. Even with an aggressive use of these techniques, the average survival time for patients with these tumors is less than two years. The tumor cells invade the normal brain tissue in a way that is not well visualized by current imaging techniques and in areas that are difficult to remove. In addition, evidence suggests that malignant gliomas are prone to genetic instability, thereby favoring tumor expression. Finally, these tumors are resistant to ionizing radiation (IR) and therefore, even after the removal of the tumor bulk the remaining tumor cells resume growth (Broaddus et al., 1999).

The most common and malignant of brain tumor is gliomablastoma multiforme. These tumors are characterized by the occurrence of necrosis, vascular proliferation, and aggressive invasion into adjacent normal brain tissue as seen in Figure 1. The diffusely infiltrative nature of glioblastoma multiforme is one of the chief obstacles to its successful surgical control (Kondraganti et al., 2000).

Figure 1:The large white area in the lower right represents a parietal glioblastoma multiforme shown by magnetic resonance imaging (MRI) (www.med.harvard.edu)

The extracellular matrix, composed of structural components such as collagens, proteoglycans, and glycoproteins, is the scaffolding in which both normal and tumor cells reside. Excessive breakdown of the extracellular matrix is associated with cancer, as well as arthritis, ulceration, emphysema, and cardiovascular disease (Nagase and Woessner, 1999). Though many enzymes have the ability to degrade components of the extracellular matrix, including cystine and serine proteases, the matrix metalloproteinase family is known to be particularly significant in matrix degradation. These enzymes are believed to play a key role in the invasiveness and metastatic ability of a variety of malignant tumors (Kondraganti et al., 2000).

The matrix metalloproteinase (MMP) family is currently comprised of twenty-four members. These enzymes have overlapping substrate specificities and collectively have the ability to degrade almost all of the components of the extracellular matrix. MMPs exist in both invertebrates and vertebrate species. In vertebrates, they are

expressed primarily in connective tissue cells and in cells that have originated in the bone marrow (Morgunova et al., 1999).

All MMPs require the binding of two zinc atoms, hence the metallo- prefix. This binding is essential for proteolytic activity (Lovejoy et al., 1994). The binding occurs in the catalytic core domain, which contains the active protein-degrading ability of the proteinase. They all contain a signal peptide for synthesis on the rough endoplasmic reticulum. All MMPs also possess the conserved sequence PRCGXPD in the propeptide domain so that the cystine can function as an activation switch mechanism (Vallee and Auld, 1990). Removal of the propeptide domain requires cleavage between the first and second helices within the propeptide bait region, as shown in Figure 2 (Morgunova et al., 1999). All MMPs, except MMP-7, have a C-terminal domain that has homology to a serum protein called hemopexin. The hemopexin domain is believed to mediate additional protein-protein interactions with substrates and with naturally occurring inhibitors (Ward et al., 1994).

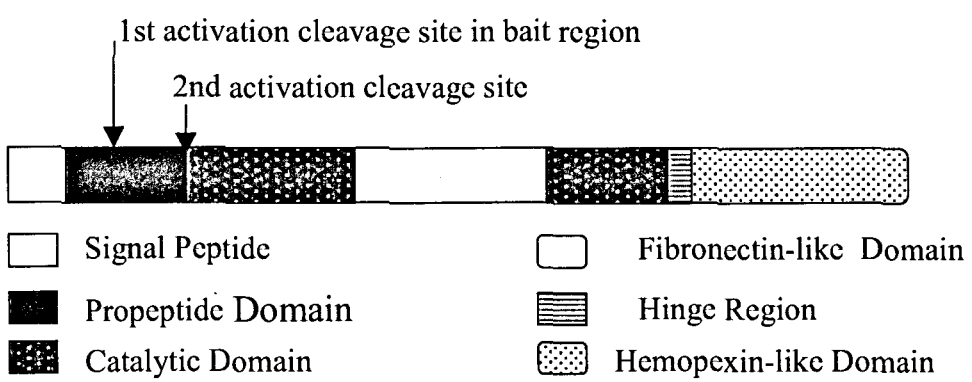

Figure 2: Detailed protein domains structure and Activation of Pro-MMP-2 of matrix metalloproteinase-2. Activation of 72kDa pro-MMP-2 occurs by the cleavage of the amino terminal propeptide in the bait region, resulting in a 64kDa conversion intermediate. A second cleavage by autoactivation results in the 62kDa active MMP-2. (Nguyen, 2000).

MMPs are classified according to the additional protein domains that contribute to their characteristics (Nagase and Woessner, 1999). The roles of additional functional domains include modulating protein interactions important in substrate recognition, providing transmembrane localization, or providing an alternative cleavage site for MMP activation (Murphy et al., 1994). The members of the MMP family have been divided into six subgroups: interstitial collagenases, gelatinases, stromelysins, metalloelastases, secreted RXKR-containing MMPs, and membrane type MMPS (MT-MMPs) (Theret et al., 1998).

MMPs are highly regulated. Expression, secretion, and activity levels are under tight control. MMPs are expressed at very low levels, if at all. At times of active tissue remodeling, expression is rapidly induced (Nagase and Woessner, 1999). The activity of MMPs is controlled extracellularly through the cleavage of the pro-domain from the inactive MMP to create the catalytically active MMP. Activity is further regulated by interactions with their natural inhibitors, the tissue specific inhibitors of matrix metalloproteinases (TIMPs). The four members of this family are collectively capable of binding and inhibiting the activity of all members of the MMP family (Gomez et al., 1997).

Matrix metalloproteinase-2 is a 72kDa type IV collagenase, also called gelatinase A. The MMP-2 gene, located at locus q21 on human chromosome 16, is 27 kilobases consisting of 13 exons and 12 introns (Huhtala et al., 1990). The introns are removed during processing to create a 6.9 kb mRNA molecule, from which a 1.98 kb structural fragment is translated into a 660 amino acid protein. The signal peptide directs pre-

proMMP-2 to the rough ER, which directs it into the secretory pathway. The signal peptide is cleaved and proMMP-2 is secreted from cells as a zymogen (progelatinase A) (Strongin et al., 1993). MMP-2 is primarily expressed in mesenchymal cells, mainly fibroblasts, during development and tissue regeneration. It was originally isolated from a malignant mouse tumor and was found to be highly expressed in stromal cells surrounding the invading front of a metastasizing tumor (Morgunova et al., 1999).

The activity of MMP-2 in the extracellular environment is controlled by various activators and the endogenous tissue inhibitors of matrix metalloproteinases (TIMPs). Activation of 72kDa MMP-2 occurs by the cleavage of the amino terminal propeptide, resulting in a 64kDa conversion intermediate with an amino terminus at N-Leu^{33}. This intermediate is converted to a N-Tyr^{81} 62kDa active form, as shown in Figure 2. Crosslinking and activation experiments demonstrate that membrane-associated TIMP-2 interacts with the carboxyl-end domain of MMP2 and is an essential part of the cell surface activation complex (Strongin et al., 1995). Recent data suggests that TIMP-2 may mediate the cell surface activation of MMP-2 by binding to an MT1-MMP-containing complex on the cell surface. The two may act together as a receptor for pro-MMP-2 and lead to the cleavage of this zymogen (Theret et al., 1998).

Matrix metalloproteinase-2 (MMP-2) is transcriptionally regulated by p53, a tumor suppressor gene and transcription factor. p53 plays an important role in determining whether a cell will undergo differentiation, senescence, or apoptosis. It stops cell cycle progression at both the G_1 checkpoint before replication of damaged genetic material and the G2/M transition. Loss of cell cycle control, due to a loss of functional

p53, is believed to contribute to tumor development. Furthermore, p53 is able to trigger DNA damage-induced apoptosis, or programmed cell death. Apoptosis is necessary for the development of the CNS as well as in tumor prevention and treatment (Fulci and VanMeir, 1999). Therefore, it appears that p53 plays a vital role in inhibiting tumorigenesis in many types of cancer.

A p53 binding site in the promoter of the human MMP-2 gene has been identified. A number of studies strongly suggest that the MMP-2 gene is a p53 target gene and that its expression is subject to p53 regulation. These studies have shown that activation of the promoter activity is p53-binding dependent in p53 positive cells but not in p53 negative cells. Only wild-type p53, not p53 mutants commonly found in human cancers, induces MMP-2 expression. Expression of endogenous MMP-2 is also under control of p53 (Bian and Sun, 1997).

A feature commonly associated with tumor invasion and angiogenesis is the degradation of the extracellular matrix, basement membrane, basal laminae, and interstitial stroma. Basement membranes contain type IV collagen as a major component as well as laminin, fibronectin, heparan sulfate proteoglycan and entactin (Shina et al., 1991). Neoplastic cells that invade surrounding tissues and go on to metastasize through the bloodstream must penetrate the basement membranes of the blood vessels in order to enter and to leave the vascular compartment, as shown in Figure 3. Therefore, the ability of neoplastic cells to degrade basement membranes is one important determinant of metastatic potential. This realization led to the discovery of gelatinase A or, now more commonly known as MMP-2, which degrades type IV collagen as well as other

components of the extracellular matrix. MMP-2 is involved in the extracellular matrix remodeling in a wide range of non-neoplastic processes, such as regulation of cell proliferation and differentiation (Liotta et al., 1979).

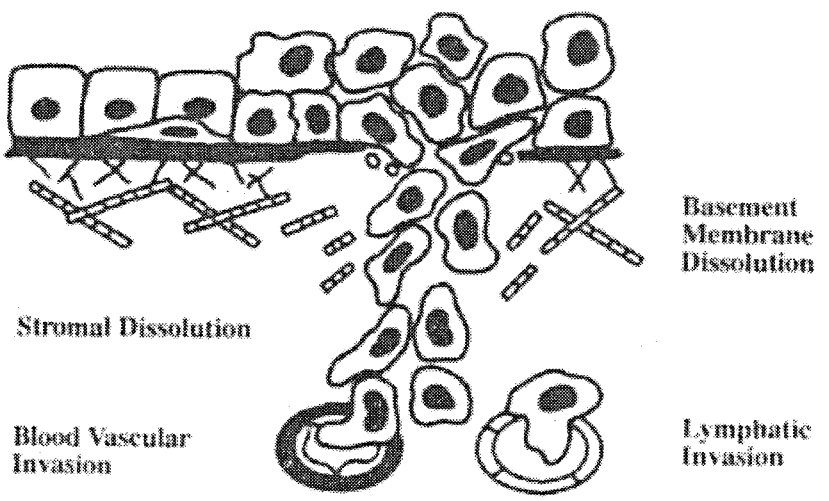

Figure 3: Destruction of the basement membrane and invasion of neoplastic cells (Liotta, 1983).

MMP-2 expression has been linked to tumor invasion. Unlike other MMPs, the activation of MMP-2 takes place on the cell surface, which confers a pivotal role in migration and growth of tumor cells (Theret et al., 1998). Angiogenesis and corresponding tumor growth have been shown to be reduced in MMP-2 knockout mice (Itoh et al., 1998). Other studies have shown the importance of activation of pro-MMP-2 for the invasion and metastasis of many types of human cancers such as gliomas, breast carcinomas, thyroid papillary carcinomas, ovarian carcinomas, gastric adenocarcinomas, and oral squamous cell carcinomas (Okada, 2000). Previous studies have investigated relative amounts of MMP-2 production in the normal brain in comparison to amounts in low-grade gliomas and amounts in more highly invasive malignant astrocytomas. Much

higher MMP-2 levels were localized to tumor and vascular endothelial cells in malignant astrocytomas in comparison to low-grade gliomas and normal brains (Lampert et al., 1998).

Evidence suggests a correlation with MMP-2 expression and glioma invasiveness. This study further investigates this correlation and looks for a more direct link between an increase in MMP-2 expression and an increase in invasion. In order to do this, it was necessary to create and characterize a mammalian expression system, which contained full length MMP-2 cDNA in the sense and antisense orientation. A sense construct is used to manipulate the cells to overproduce a specific protein, in this case MMP-2. Antisense constructs are designed to bind to a specific sequence of nucleotides in its mRNA target to inhibit production of the protein encoded by the target mRNA. In this case, antisense MMP-2 binds to the MMP-2 mRNA produced by the glioma cells, thus blocking MMP-2 synthesis as shown in Figure 4. By acting at this earlier stage in the disease-causing process to prevent the production of a disease-causing protein, antisense drugs have the potential to provide greater therapeutic benefit than traditional drugs which do not act until the disease-causing protein has already been produced.

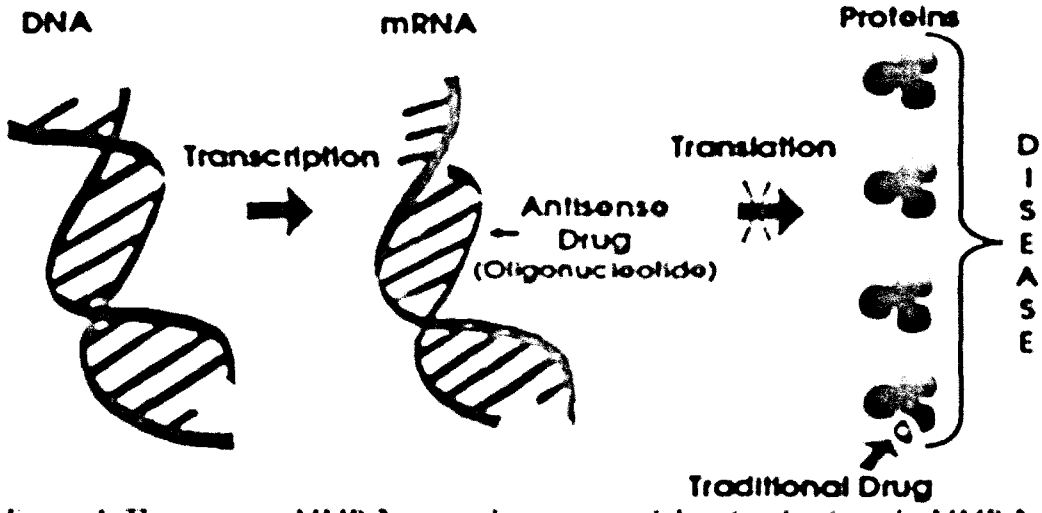

Figure 4 The antisense MMP-2 is complementary and therefore binds to the MMP-2 mRNA preventing translation and thus preventing MMP-2 protein production (http://www.isip.com/antisens.html)

Therefore, through the use of MMP-2 cDNA sense and antisense constructs, several glioma cell lines have been manipulated to overexpress and underexpress MMP-2. The use of this expression system enables further investigation of how changes in MMP-2 expression affect glioma invasion within established glioma cell lines. In this study, the invasiveness of these tumors has been evaluated to determine if the tumors that are overexpressing the enzyme are more invasive than those that are underexpressing the enzyme. The tools created in this study can not only be used to further investigate the link between MMP-2 and invasion, but it can be used to see if an increase in MMP-2 expression is in fact the major cause of glioma invasion, which is currently unknown

Materials and Methods

Maxi Prep to Extract Plasmid Constructs from Transformed *E.coli* cells

The DNA used in this experiment was extracted from glycerol stocks of MMP-2 clones received from the William Broaddus and Helen Fillmore laboratories at Virginia Commonwealth University- Medical College of Virginia. The QIAprep Spin (QIAGEN) endo-free maxiprep kit was used to extract the plasmid constructs from the TOP10F' colonies. The endo-free kit was used because lysis of bacterial cells release endotoxins which reduce transfection efficiency. Luria broth (LB) (3ml) containing kanamycin was inculated from the glycerol stock. Each culture (1ml) was grown overnight in 100ml of LB. Extraction was performed according to the manufacturer's instructions. The process of extracting the plasmid involves alkaline lysis of bacterial cells followed by adsorption of only DNA onto a silica-gel membrane. Following washes, centrifugation, and eluation steps, one obtains high recovery of pure plasmid DNA. The DNA was eluted in 500µl of TE.

Cell Culture

The cell lines used in this experiment T98, U251, LNZ-308, U373, and RT2, were obtained from the Medical College of Virginia at Virginia Commonwealth University. The cell lines were maintained in 75cm^2 flasks in Dulbeco's Modified Eagle Medium or DMEM (Gibco/BRL11965-092) with high glucose, pyridoxine HCL, and L-Glutamine. The media was supplemented with 10% fetal bovine serum (FBS) (Gibco/BRL 16000-044) that was previously heat inactivated, 1% nonessential amino acids (Gibco/BRL

11140-050), 0.1% gentamicin sulfate (BioWhittaker 17518Z) and 1% penicillin-streptomycin(Gibco/BRL 15140-122).

To pass the cells under sterile conditions, the old media was removed the cells and the cells were washed with 6.0ml phosphate buffered saline. 10X Trypsin-EDTA (Sigma T4171) diluted 1 part in 4 in serum-free media was added to the cells and they were incubated at 37°C for 3-5 minutes or until a majority of the cells were floating. The contents of the flask were then transferred to a sterile 50ml centrifuge tube and centrifuged at 1300rpm for ten minutes at room temperature. The supernatant was removed and the pellet was resuspended in 5-25ml of DMEM-FBS depending on pellet size. In order to count the cells, 1:10 and 1:1000 dilutions of the cell suspensions were made. 10µl of each diluted sample was added to each end of a hemacytometer. The cells were counted in four corners of the grid and an average was taken. The average number of cells was multiplied by 10,000 and then by the dilution factor to obtain the total number of cells per ml. The desired number of cells was then added to a flask containing DMEM-FBS.

U251, U373, RT2 and LNZ cell lines were stably transfected with the MMP-2 gene in the sense and antisense orientation through previous work conducted at the Medical College of Virginia. The cells were also transfected with the control pCR3.1 vector. These cells were maintained in a specific concentration of Geneticin (the brand name of G418 from Gibco/BRL 10131-035) determined by a kill curve. This procedure selects only stably transfected cells. All of the cells are kept in an incubator at 5% CO_2, 95% humidity and 37°C.

Creating a G418 Concentration Kill Curve

Prior to transfection it was necessary to determine the concentration of G418 to use for tranfectant selection. The concentration of G418 must be just high enough to kill all cells that did not pick up the pCR3.1 vector during transfection. A G418 kill curve was generated with increasing concentration of Geneticin for the cell line T98. G418 is an analog of the neomycin aminoglycoside that interferes with the function of 80S ribosomes and blocks protein synthesis in eukaryotic cells. The neomycin resistance gene encodes a protein that transfers a phospho-, acetyl, or nucleotidyl- group onto the aminoglycoside, thus inactivating it. Each cell line was seeded at $1X10^5$ cells/well in a 24 well plate. After reaching 80-100% confluence, 0.25mg/ml to 2mg/ml of G418 was added. The cells were monitored over four days. The lowest concentration of G418 that left the majority of cells dead and floating was the concentration chosen to use for selection of transfected cells.

Transfection of Cell Lines with Sense and Antisense MMP-2 and Control Vector

The cell lines were transfected using Lipofectamine 2000 (Gibco/BRL 11668-019). Lipofectamine 2000 is liposome-mediated means of transferring DNA into mammalian cells. With cationic lipid reagents, the negatively charged DNA binds spontaneously to the positively charged liposomes, forming DNA-cationic lipid reagent complexes. The liposome complexes fuse with the plasma membrane and the captured DNA is then delivered into the cytoplasm of the cultured cells. Cells were plated in 24-well plates the

day before transfection at a concentration of $4X10^5$ cells/ml. Twenty-four hours later, 1.0µg of DNA per well was diluted in 50µl of DMEM-FBS lacking antibiotics. 2.5µl of Lipofectamine 2000 was diluted in 50µl of DMEM-FBS also lacking antibiotics. Antibiotics were excluded from the media because the cationic lipid reagents increase the cells' permeability and allow delivery of antibiotics into the cells. This decreases the health of the cells, thus lowering transfection efficiency. The diluted DNA and diluted lipofectamine were combined in microfuge tubes and incubated for twenty minutes at room temperature. The complexes were then added to the cells, which were then incubated for twenty-four hours. Transfection control cells were given DNA or lipofectamine. Twenty-four hours after transfection the cell lines were passed to 6-well plates. Twenty-four hours after the cells were passed, G418 was added to the cells at a concentration of 1.0 mg/ml (determined by a kill curve, to select for the transfected cells). The cells were observed daily for the formation of colonies. Cloning rings and trypsin-EDTA (Sigma) were used to isolate and pass each colony to individual wells.

Determining Protein Concentration Using the Bradford Assay

Bradford assays were conducted on all protein samples prior to zymography or Western blot analysis to standardize protein concentration. BioRad Dye Reagent was diluted 1:5 in superwater and filtered using Whatman No.1 filter paper. 100µl of sample was added to 5ml of dye and allowed to sit for 5 minutes. Absorbance was read at 595nm on a Spectronic 21. Protein concentration was determined by the standard curve equation Slope =0.011A/µg .

Determining MMP-2 Activity Via Zymography

The transfected cell lines were analyzed for MMP-2 activity induction by zymographic substrate gels. Zymogram gels (NOVEX EC6175) are 10% Tris-Glycine gels copolymerized with 0.1% gelatin such that the activity of the enzyme can be assessed within the gel. The transfected cell lines were incubated with serum free media for 48 hours then the media was removed. Protein concentrations of each sample were standardized by a Bradford assay. 15µl of the samples were mixed with 15µl Tris-Glycine SDS Sample Buffer (NOVEX LC2676). The mixtures were allowed to stand for 10 minutes at room temperature. The gels were run at 120 volts for 90 minutes with 1X Tris-Glycine SDS Running Buffer (NOVEX LC2675). The gel was then incubated in 1X Renaturing Buffer (NOVEX LC2670) for thirty minutes at room temperature and then equilibrated in 1X Developing Buffer (NOVEX LC2671) at room temperature. Following staining for thirty minutes with 0.1% Coomassie Blue, gels were destained with 10% isopropanol and 10% glacial acetic acid approximately one hour or until clear bands could be seen. The gel was then visualized on a light box.

Determining MMP-2 Expression Via Western Blot Analysis

Western blots were used to analyze cell lines for the production of pro-MMP-2. The transfected cell lines were incubated with serum free media for 48 hours and the media

was removed. Protein concentrations of all samples were standardized by a Bradford assay. 20µl of each sample was added to 4X Sample Buffer (NuPAGE NP0007). 2.5µl of Reducing reagent (NuPAGE NP0004) was added to each sample and the samples were incubated at 70°C for ten minutes. The samples were loaded into a Bis-Tris-HCl buffered polyacrylamide gel (NuPAGE NP0321) containing 1X Running Buffer (NuPAGE). 200µl of antioxidant was added to the buffer filling the inner buffer chamber. The gel was run at 200 volts for 35 minutes. Transfer buffer was prepared by diluting Tris Glycine Transfer Buffer (NOVEX, NP0005) with deionized water and 10% methanol. The transfer buffer was kept ice cold. Blotting pads were soaked in transfer buffer. A nitrocellulose membrane was soaked in deionized water then transfer buffer. Two pieces of filter paper were soaked in transfer buffer immediately prior to use. The gel membrane apparatus was assembled with two blotting pads in the cathode core, followed by a piece of filter paper, followed by the gel and nitrocellulose membrane, then two more blotting pads. The chamber was filled with ice-cold transfer buffer and the transfer was run at 30 volts for 1 hour. The membrane was removed from the transfer apparatus and put in a hybridization bottle with the protein side facing the interior. It was then blocked in blocking solution (consisting of 3.0% nonfat dry milk and TBS) for 2 hours at room temperature. It was then washed in 1X TBS (consisting of 1.54M NaCl and 0.1M Trizma) for five minutes. A 1:2000 dilution of rabbit MMP-2 primary antibody (Chemicon AB809) in TBST (consisting of TBS, 0.1% Tween, and 0.2% nonfat dry milk) was then added to the blot for 2 hours at room temperature. It was then washed three times for ten minutes each in TBST. A 1:5000 dilution of horseradish peroxodase

conjugated goat anti-rabbit secondary antibody (Rockland 611-1302) in TBST was added to the blot for 1 hour at room temperature. The blot was then washed 4 times with 10ml TBST for five minutes each. The membrane was incubated with 7 ml ECL detection reagents for one minute 1:1 v:v;0.125ml/cm^2 membrane. The reagent was drained from the membrane and it was smoothly wrapped in saran wrap and developed in the dark room. The Polaroid film was exposed for one minute, developed for 2 minutes without agitation, soaked in stop bath for 30 seconds with agitation, soaked in fixer for 2 minutes with intermittent agitation and then washed under running water.

Fluorescent labeling of Cells

Cells were fluorescently labeled by DiI (Molecular Probes, V-22885), a long-chain dialkylcarbocyanine that uniformly labels through lateral diffusion into the plasma membrane. Cells were grown up in 6 well plates to approximately 90% confluence, then were washed with PBS. DiI was diluted 1:200 in 1mL serum free media. 2mL of diluted DiI was added to each well of cells. The cells were incubated with DiI overnight. The media was then removed and the cells were washed with PBS three times. The cells were then trypsinized and counted.

Invasion Assay

An *in vitro* invasion assay was conducted to determine whether the cell lines that are overproducing MMP-2 are in fact more invasive. Matrigel basement membrane matrix (Becton Dickinson) extracted from Englebreth-Holm-Swarm (EHS) mouse tumor can be

used as a reconstituted basement membrane *in vitro* that provides a biologically active extracellular matrix. The matrix has been shown to contain collegen IV, laminin, entactin, heparan sulfate, and proteoglycan. The 204µl matrigel matrix was diluted in 1.796ml serum free DMEM. 50µl was added to each transwell permeable growth support (COSTAR 3422). The plate was then be incubated for 40 minutes at 37°C to allow binding. The media was then removed from the inserts and they were washed with DMEM. Then 50µl of a concentration of $1X10^5$ cells/ml was added to each insert. DMEM was added to the lower compartment to serve as a chemoattractant. Invasive cells attach to and invade the matrix, pass through the membrane pores, and emerge on the underside of the membrane, while non-invasive cells do not migrate through the occluded pores.

Plate Reading

Invasion assay plates were read every 24 hours for five days. Fluorescence was read by BMG Labtechnologies FLUOstar Galaxy. The excitation filter was set at 544nm and the emission filter was set to 590nm.

Results:

At the Broaddus/Fillmore Laboratories at Virginia Commonwealth University-Medical College of Virginia, MMP-2 was cloned and ligated into the bi-directional pCR3.1 vector as seen in Figure 5. Competent *E.coli* cells were transformed by heat shock. The SV40 early promoter and the neomycin/kanamycin resistance gene allowed for selection of transformed *E.coli* colonies. Restriction enzyme analysis was used to determine the presence and orientation of MMP-2 in pCR3.1 vector. Automated Fluorescent Dideoxy Sequencing was used to verify orientation and sequence of the cloned genes. One clone containing MMP-2 in the sense orientation, antisense orientation, and the control vector were frozen in glycerol for further use.

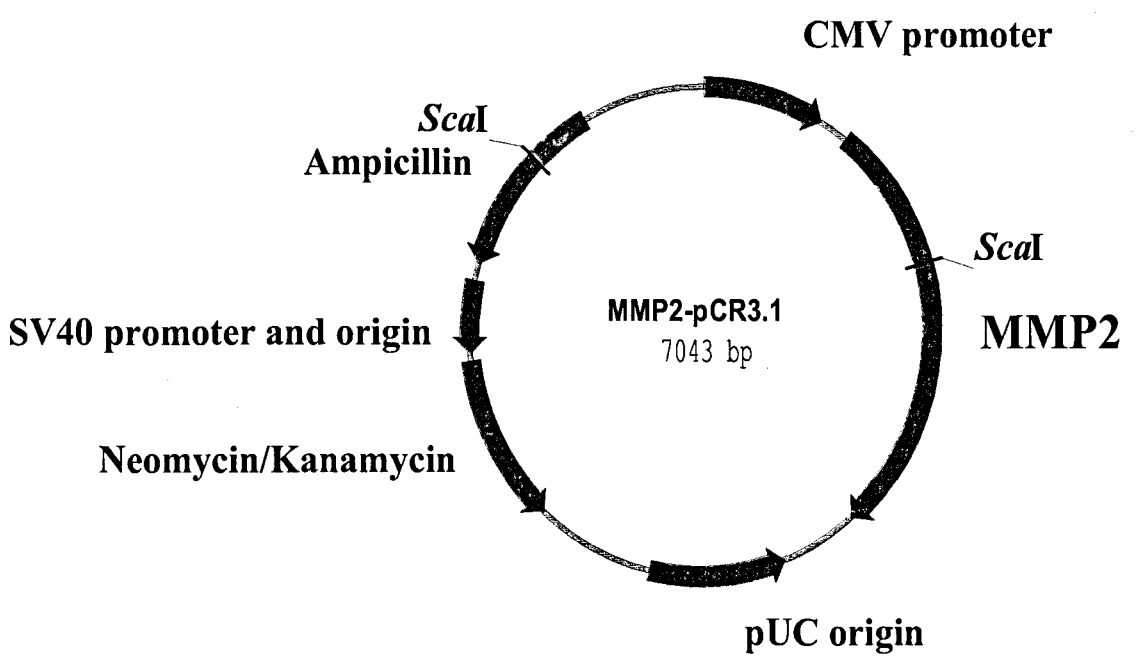

Figure 5: MMP-2 cDNA was inserted in the sense and antisense orientation into a pCR3.1 vector with a CMV promoter for high-level expression. Glioma cell lines were transfected with sense/control/antisense DNA constructs.

Maxi Prep

The glycerol stocks were obtained from the Broaddus/Fillmore laboratories to extract the plasmid DNA to be used in transfection. QIAprep Spin endo-free maxiprep kit was used to extract the plasmid constructs from the TOP10F' *E.coli* cells. The DNA concentrations were determined by UV spectrophotometry as 345ng/µl control DNA, 271.5ng/µl sense DNA, and 586ng/µl antisense DNA. A 1% agarose gel was run at 90 volts for 30 minutes to ensure the integrity of the DNA. Figure 6 shows that the plasmid DNA is intact.

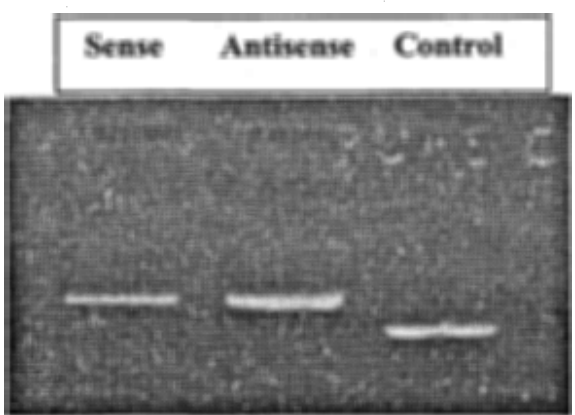

Figure 6: 1% agarose gel to determine plasmid DNA integrity.

Kill Curve

Prior to transfection it was necessary to determine the concentration of G418 to use for tranfectant selection. The SV40 early promoter and the neomycin/kanamycin resistance gene in the pCR3.1 vector allowed for selection of stably transfected cell lines.

A G418 kill curve was generated with increasing concentrations of Geneticin for the cell line T98 as shown in Table 1. The cells were observed over 4 days.

Concentration of G418 (mg/ml)	Cell Culture Observations
0.00	All cells alive and healthy
0.25	All cells alive and healthy
0.50	Some cells dead and floating, some healthy cells
0.75	Most cells dead and floating; few adherent cells no longer have processes and appear unhealthy
1.00	All cells are dead and floating
1.25	All cells are dead and floating
1.50	All cells are dead and floating
1.75	All cells are dead and floating
2.00	All cells are dead and floating

Table 1: Observations of T98 cells after 4 days of treatments with designated concentrations of G418.

The ideal concentration of G418 to be used for transfection selection is the concentration that is just high enough to kill all cells that did not pick up the pCR3.1 vector during transfection. The lowest concentration of G418 that killed all of the T98 cells was 1.00mg/ml. This concentration was used to select for transfected T98 cells.

Transfection

The next step of this study was to establish several stably transfected glioma cell lines. Each glioma cell line was transfected with MMP-2 in the sense orientation, MMP-2 in the antisense orientation, and pCR3.1 vector to serve as a control by cationic lipid reagent-mediated transfection. Following transfection, the concentration of G418 determined by the kill curve was added to the cells for selection. Cells that survived

selection were allowed to grow up into large macroscopically visible colonies. Several colonies resulted from each transfection and were differentiated by number as shown in Table 2. These colonies were then isolated by the use of cloning rings as shown if Figure 7. Each of these colonies resulting from transfection was grown up until there were enough cells present to collect conditioned media from the cells to be analyzed for MMP-2 production.

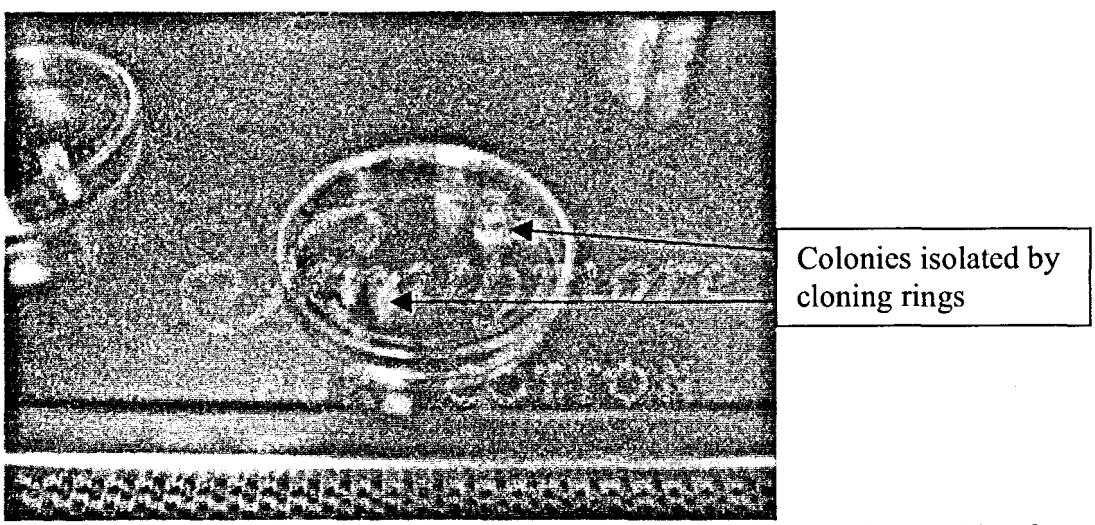

Figure 7: Cloning rings dipped in celloseal were placed over the colonies resulting from transfection. Each transfection resulted in several colonies. The colonies were collected and expanded to separate plates to allow each colony to grow.

	U251			T98			LNZ-308	
Sense	Antisense	Control	Sense	Antisense	Control	Sense	Antisense	Control
5	1	1	1	1	1	1	2	1
	6		2	2			5	
			3	3				

Table 2: Colonies resulting from transfection analyzed by zymography and Western blot analysis

Zymography and Western Blot Analysis

Once the transfected cells had grown to confluence, it was essential to test the MMP-2 status to be sure that the transfection worked properly and to screen the cells for use in further use in invasion testing. The MMP-2 production of the transfected cell lines was characterized through the use of Western blots to analyze MMP-2 induction and zymography to analyze MMP-2 activity. It was hypothesized that little to no MMP-2 induction or activity would be present from the cells transfected with the control or antisense MMP-2 and increased expression and activity of MMP-2 would be present from the cells transfected with the sense MMP-2.

For zymography and Western blot analysis, conditioned media was collected from the cells to analyze MMP-2 secreted from the cells. The transfected cell lines were incubated with serum free media for 48 hours and the conditioned media was removed. Protein concentrations of all conditioned media samples were standardized by a Bradford assay. Assays were run with 25μg of protein as soon as possible after collection from the cells.

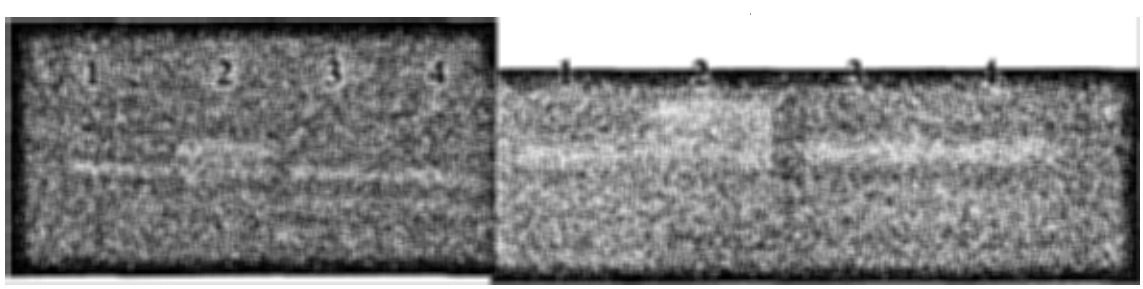

Figure 8: Zymograms of U251 Conditioned Media Samples. Conditioned media samples were assayed 24 hours after collection from U251 cells. Each gel represents data from two experiments. In each gel, lane 1 control clone #1, lane 2 sense clone #5, lane 3 anti-sense clone #1, and lane 4 anti-sense clone #6. All lanes were loaded with 25μg protein determined by a Bradford assay.

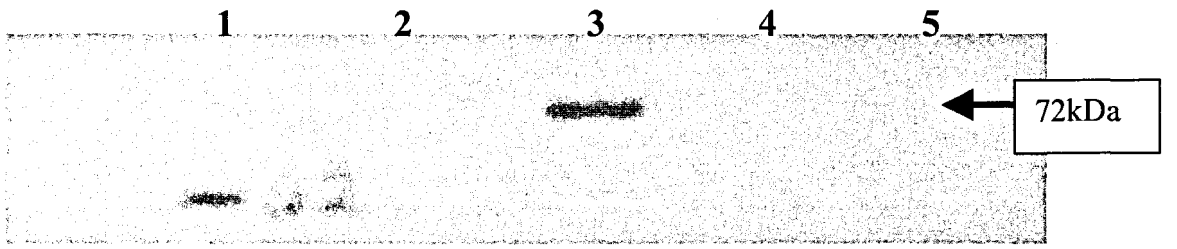

Figure 9: Western Blot Analysis of U251 Conditioned Media Samples. Conditioned media samples were assayed 2 hours after collection from cells. Lane 1 contains a molecular weight marker, lane 2 control clone #1, lane 3 sense clone #5, lane 4 anti-sense clone #1, and lane 5 anti-sense clone #6. All lanes were loaded with 25μg protein determined by a Bradford assay.

Zymograms and Western blots were conducted in triplicate on the U251 cells to analyze MMP-2 activity and expression respectively. Representative data from these experiments is shown in Figures 8 and 9. Both zymogram gels in Figure 8 show that the U251 sense 5 cells appear to be overproducing gelatin degrading enzymes such as MMP-2 (lane 2). The control in lane 1 and the antisense cells in lanes 3 and 4 seem to be exhibiting equal expression. Analysis by zymography is necessary to show the gelatinase activity of the cells, however, this activity not necessarily due solely to MMP-2. The activity shown from the series of bands on the zymograms is a result of all gelatin-degrading enzymes present in the cells. However, the additional upper band seen if Figure 8 from only the sense cells is due to the presence of transfected sense MMP-2.

In Western blot analysis of the same samples shown in Figure 9, a clear band indicating MMP-2 expression can be seen from the U251 sense sample 5 (lane 3). According to molecular weight markers, this band is approximately 72 kDa, the size of

pro-MMP-2. No bands were in the U251 control and sense samples. Similar results were seen from all of the characterization experiments conducted on the U251 cells.

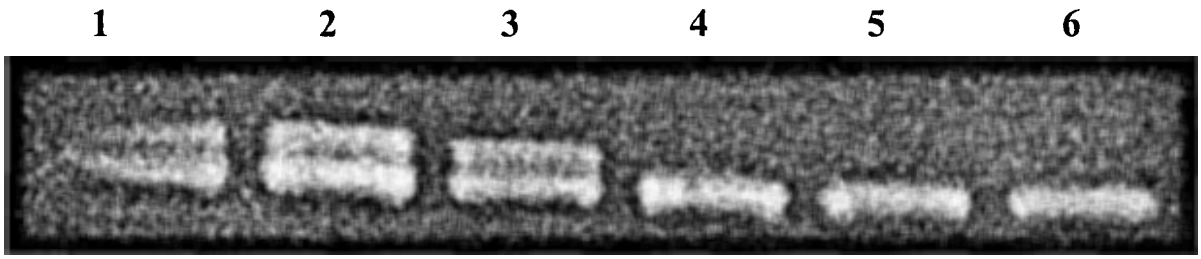

Figure 10: Zymogram of T98 Conditioned Media Samples. Conditioned media samples were assayed 24 hours after collection from cells. Lane 1 contains the T98 sense clone #1, lane 2 sense clone #2, lane 3 sense clone #3, lane 4 control clone #1, lane 5 anti-sense clone #1, lane 6 anti-sense clone #2. All lanes were loaded with 25μg protein determined by a Bradford assay.

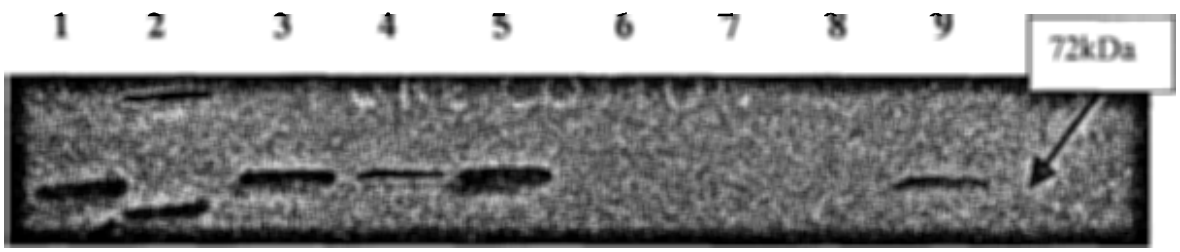

Figure 11: Western blot analysis of T98 Conditioned Media Samples. Conditioned media samples were assayed 1 hour after collection from cells. Lane 1 contains the positive control MMP-2 protein, lane 2 the molecular weight markers, lane 3 contains T98 sense clone #1, lane 4 contains sense clone #3, lane 5 contains sense clone #2, lane 6 contains T98 control clone #1, lane 7 contains anti-sense clone #1, lane 8 contains anti-sense clone #2, lane 9 contains anti-sense clone #3. All lanes were loaded with 25μg protein determined by a Bradford assay.

Zymograms and Western blots were conducted in triplicate on the T98 cells to analyze MMP-2 activity and expression. In the zymogram shown in Figure 10 from the T98 transfected cells, gelatin degradation was present in each of the samples, however, a

double band was seen from the sense # 1, sense #2, and sense #3 cells in lanes 1, 2, and 3. The lower band that is present from all of the cells may or may not be due to MMP-2 alone, however the upper band seen from the sense cells is an increase in gelatinase activity due to transfection with sense MMP-2.

In the Western blot analysis shown in Figure 11, clear 72kDa bands representing the presence of MMP2 can be seen in all of the sense cells (lanes 3-5). The strongest band can be seen from the sense clone #2 in lane 5. A band is also seen from the antisense clone #3 in lane 9. These cells were discarded because T98 cells transfected with antisense MMP-2 should not be overproducing MMP-2. Repeatable results were seen from the Zymograms and Western blots as these tests were conducted in triplicate.

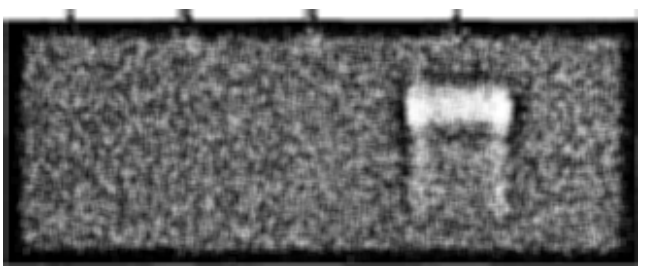

Figure 12: Zymogram of LNZ-308 Conditioned Media Samples. Conditioned media samples were assayed 1 hour after collection from cells. Lane 1 contains LNZ-308 control clone #1, lane 2 antisense clone #2, lane 3 antisense clone #5, and lane 4 sense clone #1. All lanes were loaded with 25μg protein determined by a Bradford assay.

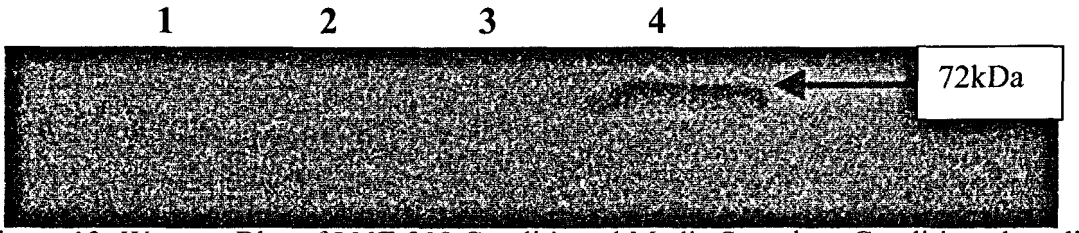

Figure 13: Western Blot of LNZ-308 Conditioned Media Samples. Conditioned media samples were assayed 24 hours after collection from cells. Lane 1 contains LNZ-308 control clone #1, lane 2 antisense clone #2, lane 3 antisense clone #5, and lane 4 sense clone #1. All lanes were loaded with 25μg protein determined by a Bradford assay.

Zymograms and Western blots, Figures 12 and 13, were conducted in triplicate on the LNZ-308 cells to analyze MMP-2 activity and expression respectively. In the LNZ-308 cells, an increase activity can be seen from the sense cells in lane 4 of the zymogram in Figure 12. Since no gelatinase activity is seen from the control and the antisense cells, the activity seen from the sense cells is due to transfection with sense MMP-2. A clear band corresponding to 72kDa MMP-2 is present in lane 4 of the Western blot in Figure 13. No MMP-2 production is apparent from the control or antisense cells in Figure 13.

Invasion assay

Subsequent to characterizing MMP-2 activity of the U251 cells, an *in vitro* invasion assay was conducted to determine if the cells that exhibit increased expression of MMP-2 are more invasive *in vitro* than the control and antisense transfected cell lines. Florescently labeled cells were added to Matrigel coated inserts in quadruplicate for each cell type. Due to previous studies linking MMP-2 to tumor invasiveness, it was predicted that the increase of production and activity of MMP-2 produced by the glioma cells

transfected with sense MMP-2 would lead to increased invasion by these cells. It was also predicted that the cells transfected with the antisense MMP-2 constructs would block MMP-2 induction and show a decrease in invasion.

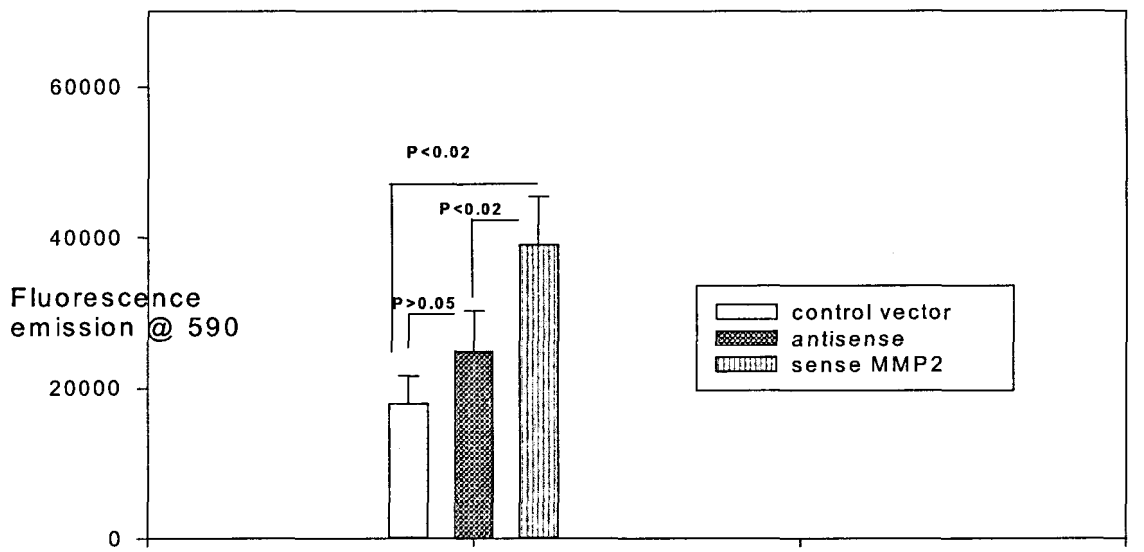

Figure 14: U251 stably transfected with control vector, antisense MMP-2 and sense MMP-2. The florescence of the cells that had invaded the Matrigel coated transewell inserts and ended up in the bottom of the wells was measured at a fluorescence emission of 590nm after 72 hours. The total fluorescence for each of the four wells was averaged for each cell type. T-tests were run to determine statistical significance.

In the invasion assay in Figure 14, the amount of fluorescence measured on the bottom of each well is indicative of the degree of invasion of each cell type. A significant difference in invasion can be seen in comparing the fluorescence of the sense cells to the control cells, and in comparing the sense cells to the antisense cells. No significant difference is seen in comparing the amount of fluorescence between the control and antisense cells. These preliminary results from the *in vitro* invasion assay

show significantly more invasion from the sense cells that are overproducing MMP-2 than the control and antisense cells.

Discussion

Cancer is a disease whose hallmark is uncontrolled growth and metastasis. It is based on a loss of normal growth control that produces a growing tissue mass known as a tumor or neoplasm. Malignant tumors are capable of spreading by invasion and metastasis, which involves invasion of surrounding tissues and vessels, transport via the circulatory system, and reinvasion and growth at a distant sight.

Gliomas are brain tumors containing cells that are highly invasive. Degradation of the extracellular matrix is necessary for this invasion to occur. Considerable evidence has accumulated to directly implicate members of the matrix metalloproteinase family in this process. MMP-2 protein expression has been directly linked to glioma tumor grade, where an increase MMP-2 mRNA expression is seen in the more invasive high-grade gliomas (Lampert et al., 1998). This correlative study implicating MMP-2 with invasion led to further investigation of this topic. Kodera and colleagues have found an increase in pro-MMP-2 in rat brains implanted with rat glioma cells in comparison to normal rat brains (Kodera et al., 2000). Noda and his colleagues tested a matrix-metalloproteinase inhibitor SI-27 on several human glioma cell lines, including U251 and U373. SI-27 was shown to suppress MMP-2 and MMP-9 expression and to reduce the number of invading cells migrating through invasion assay inserts (Noda et al., 2000).

These recent studies clearly demonstrate a correlation between MMP-2 expression and glioma invasion. However, there needs to be a direct link showing that modulating MMP-2 levels results in changes in glioma invasion. Therefore, the purpose of this study was to further investigate the correlation between MMP-2 expression and tumor invasion and to demonstrate that MMP-2 overexpression is the major cause of glioma invasion.

The first step of this research was to transfect several different human glioma cell lines with MMP-2 cDNA sense and antisense constructs. The plasmid DNA used in this experiment was produced from glycerol stocks of MMP-2 clones received from the W. Broaddus and H. Fillmore Laboratories at Virginia Commonwealth University Medical College of Virginia. There they conducted zymography and Western blot analysis of the non-transfected human cell lines U87, T98, U251, U373, and rat RT2, to determine endogenous MMP-2 expression. The U87 cell line was found to have high endogenous expression in comparison to the other glioma cell lines. Therefore, total RNA was extracted from this cell line to clone the entire MMP-2 gene by RT-PCR. This clone was inserted in a sense and antisense orientation into a pCR3.1 vector with a CMV promoter for high-level expression. The constructs were sequenced to determine orientation and sequence of the cloned genes. The sense and antisense constructs, as well as the pCR3.1 construct alone as a control, was used to transfect the glioma cell lines (Nguyen, 2000). A kill curve was conducted to determine the concentration of G418 to be used for transfection selection. The results from table 1 show a concentration of 1.0 mg/ml as an ideal concentration for selection of transfected cells.

The transfected cell lines were then characterized using zymography and Western blot analysis of conditioned media samples. These tests show the expression and activity of MMP-2 released from the cells. A significant difference in MMP-2 expression was seen in the zymograms and Western blots resulting from the U251, T98 and LNZ-308 cell lines. The U251 zymogram shows a clear overproduction of gelatin degrading enzymes from the cells transfected with sense MMP-2. The upper band seen in Figure 8 from the cells transfected with sense MMP-2 must be due to MMP-2 activity since it is not present in the cells transfected with antisesense or control MMP-2. The cells transfected with antisense MMP-2 and the cells transfected with control MMP-2 exhibit equal expression, but in a lesser quantity than the sense cells. It is interesting that the increase in MMP-2 activity exhibited on the zymograms appears as an additional upper band as seen from the cells transfected with sense MMP-2 in Figure 8. Since pro-MMP-2 is a 72kD protein and active MMP-2 is 62kDa, one would have predicted the increase in MMP-2 activity to appear as a band of lower molecular weight, corresponding to the 62kDa active form of MMP-2. It is possible that the cells transfected with sense MMP-2 are exhibiting so much proteolytic activity that a column of bands resulted. Since molecular weight markers do not appear clearly on the zymogram gels after staining with Coomassie blue, it is difficult to determine the exact molecular weight of the bands present in the zymogram.

In Western blot analysis of the same samples using an MMP-2 specific antibody, a clear band of 72kDa, correlating with the known molecular weight of pro-MMP-2, can be seen from the U251 cells transfected with sense MMP-2, indicating MMP-2

expression as seen in Figure 9. Little to no MMP-2 expression was seen in the samples taken from the U251 cells transfected with antisense or control MMP-2 on the Western blots. From these experiments, it is evident that transfection with sense MMP-2 lead to an overproduction of MMP-2 in these cells, while transfection with control and antisense MMP-2 did not lead to an increase in MMP-2. Since there were no differences seen in either the Western blots or zymograms from the U251 control and antisense, we cannot determine if transfection with antisense MMP-2 has blocked the induction of MMP-2.

Results similar to those seen in the U251 cell line were observed in the T98 cell line. Zymography of the samples taken from the T98 transfected cells showed gelatinase activity in each of the samples, however, the greatest activity was seen in the all of the cells transfected with sense MMP-2 as seen if Figure 10. As in the U251 samples, the enzyme activity appears as an additional higher molecular weight band that is absent in the samples from the cells transfected with control and antisense MMP-2. In the Western blot analysis, Figure 11, a 72kDa band representing the presence of MMP2 was seen in each of the samples from the cells transfected with sense MMP-2 indicating an increase in MMP-2 expression in these cells. Little to no expression of MMP-2 from the antisense and control cells was evident from Western blot analysis. The T98 antisense clone #3 that exhibited increased MMP-2 expression on the Western blot was not used in further experiments since increased MMP-2 expression was not expected from these cells. No difference in MMP-2 expression was seen when comparing the cells transfected with antisense MMP-2 and the cells transfected with control MMP-2, so just as in the U251 cells, it remains to be seen if the antisense MMP-2 is blocking MMP-2 induction in the

T98 cells. The results do illustrate that transfection of T98 with sense MMP-2 caused an overproduction of MMP-2 in these cells.

Results analogous to those seen in the characterization of U251 and T98 transfected cells can also be seen in the LNZ-308 transfected cells. As shown in Figure 12, gelatinase activity was seen in only the cells transfected with sense MMP-2, not those transfected with control or antisense MMP-2. In these cells, the degradation appears as a smear, not the distinct bands observed in the T98 and U251 samples from the cells transfected with sense MMP-2. The differences in gelatin degradation between these cell lines would be interesting to explore further.

An overproduction of MMP-2 was observed in the cells transfected with sense MMP-2 in the Western blot analysis in Figure 13. No MMP-2 production was apparent from the cells transfected with control vector or antisense MMP-2. Therefore, the LNZ-308 cells transfected with sense MMP-2 exhibited an overproduction of MMP-2, while no detectable differences in MMP-2 expression could be seen in the cells transfected antisense MMP-2 and control MMP-2 using zymography and Western blot analysis for characterization.

As a whole, the results from the characterization of MMP-2 activity by zymography and MMP-2 expression by Western blot analysis for the cell lines U251, T98, and LNZ-308 supported the hypothesis that transfection with sense MMP-2 would lead to an overproduction of this enzyme. These transfected cell lines can now be used in future experiments to relate the affects of this overexpression of MMP-2 on invasion.

Two other glioma cell lines transfected at the Broaddus/Fillmore Laboratories, RT2 and U373, were also characterized through zymography and Western blot analysis. Results from the RT2, a rat glioma cell line, showed no differences in MMP-2 expression between the cells transfected with sense, antisense and control MMP-2 in both Western blots and zymograms. It is possible that these cells lost the MMP-2 DNA during cell culture. These cells were not tested further. Human U373 cells were also characterized in a similar manner. Both of these tests showed greater MMP-2 expression from the cells transfected with control MMP-2 than those transfected with sense MMP-2. These cells may have not been transfected properly. Due to the unexpected results from these tests, caused possibly by loss of the plasmid or unsuccessful transfection, and the number of cells lines that were already being studied, these cells were no longer tested.

In future studies involving characterization of MMP-2 expression and activity in glioma cells, it may be useful to create an assay that could quantify MMP-2 expression and activity. This would be particularly valuable in determining if the cells containing the antisense construct are truly blocking MMP-2 induction and to what degree. A further means of testing the MMP-2 blocking ability of the antisense construct would be by transiently transfecting the cells that have been previously transfected with sense MMP-2, with the antisense construct. Our results have shown that the cells transfected with sense MMP-2 show an increase in MMP-2 expression and activity. If the antisense construct were working, a decrease in MMP-2 expression and activity would be predicted. Another useful experiment would be to develop a means of differentiating pro-MMP-2 from active MMP-2 by a method other than molecular weight. This could be

done by the use of different antibodies specific for different portions of pro-MMP-2 and MMP-2.

After characterizing the MMP-2 status of the transfected cell lines, it was important to test the invasiveness of these transfected cells to see if the cells transfected with sense MMP-2 exhibited and increase in invasion *in vitro*. Hotary and colleagues examined the *in vitro* invasion process of epithelial cells using Matrigel, which provides a reconstituted basement membrane to study cellular invasion through extracellular matrix components (Hotary et al., 2000). In such invasion assays conducted in the past, it was necessary to fix and stain the cells before counting them. This was a tedious process with questions arising about the accuracy and reproducibility of the results. In addition, cells could not be counted at multiple time intervals. I have developed an assay using fluorescent-labeled glioma cells. By labeling the cells with DiI, a highly lipophilic carbocyanine dye, I am able to count invading cells in real time. In addition, I believe this system provides a more accurate and reproducible assessment of invading cells than the manual methods.

Using fluorescent-labeled cells, results from the U251 invasion assay show a statistically significant difference in invasion in comparing the fluorescence of the cells transfected with sense MMP-2 to the cells transfected with control MMP-2, and in comparing the cells transfected with sense MMP-2 to the cells transfected with antisense MMP-2. No significant difference is seen in comparing the amount of fluorescence between the control and antisense cells. These preliminary results show significantly more invasion from the cells transfected with sense MMP-2 that are overproducing

MMP-2 than the cells transfected with control and antisense MMP-2, in support of my hypothesis.

I also hypothesized that there would be significantly less invasion from the U251 cells transfected with antisense MMP-2 than the cells transfected with control MMP-2. This hypothesis was not supported by the results. The fact that we see no difference in invasion between the control and antisense cells could be due to a number of reasons. First, it is possible that the antisense MMP-2 is not blocking MMP-2 induction. This could be examined by a test that quantified MMP-2 induction. It is possible that full-length antisense MMP-2 RNA is so long that it provides the opportunity for the antisense RNA to form secondary structure which would not allow it to bind to the MMP-2 mRNA, thus not blocking MMP-2 induction. It could be corrected by creating an antisense construct that contained only a portion of the antisense MMP-2 in opposed to the entire gene. The characterization experiments using Western blot or zymography have not shown any difference in MMP-2 expression between the control and antisense cells. In future experiments it would be beneficial use an assay for characterization of MMP-2 production that could quantify concentrations of MMP-2, such as an ELISA. By such a test, we would know if the antisense is truly working and to what degree it is blocking MMP-2 induction.

An invasion assay, similar to one described for the U251 cells, was conducted by the Broaddus/Fillmore laboratories on the U87 glioma cell line, as shown in Figure 15. These cells were transfected with sense and antisense MMP-2 constructs as well as the control vector. In this assay the cells were not fluorescently labeled, but instead the cells

were fixed, stained, and counted by manual methods. The results from this assay support the results seen in the U251 assay. The U87 cells transfected with sense MMP-2 were significantly more invasive than the cells transfected with the antisense or control. In this assay however, the invasion of U87 antisense cells was significantly higher than that of the control cells (Fillmore, personal communication). The reason for this is unknown. It leads to the thought that if the antisense MMP-2 is in fact blocking MMP-2 induction as it was designed to do, what repercussions could occur. If MMP-2 is knocked out, what else could be affected? It is also possible that with the addition of conA to activate pro-MMP-2, the opposite results may be seen. In addition, it would be useful to repeat this assay using the fluorescent method to see if the results are duplicated.

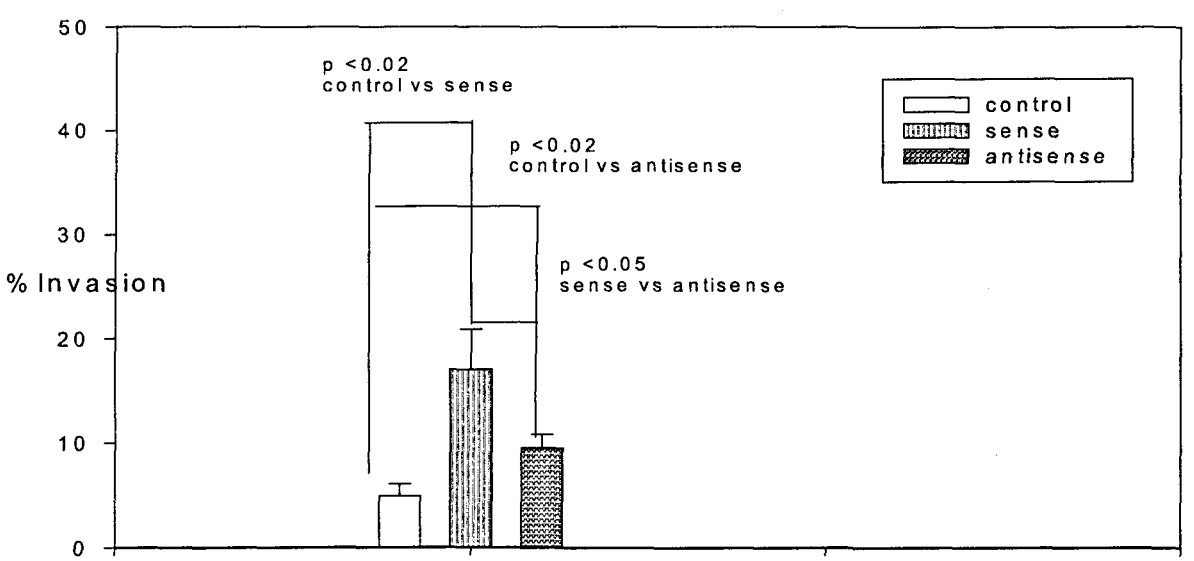

Figure 15: U87 MMP2 stable transfectants Invasion assay on U87 human glioma cells transfected with sense, antisense, and control construct conducted by the Broaddus/Fillmore laboratories at MCV. Invasion assay was run for 48 hours and cells were fixed, stained and counted to determine % invasion. T-tests were run to determine statistical significance (Fillmore, personal communication).

It is also possible that in activating MMP-2, by the tetravalent lectin concanavalin A (conA), known to induce a marked activation of pro-MMP-2 (Gingras, 2000), a difference in invasion between the control cells and the sense cells could be seen. In such an experiment, I predict that a large increase in invasion by the cells transfected with sense MMP-2 and a significantly less invasion would be seen from the antisense cells. Conducting such an assay would be an important next step in the continuation of this research.

Other future work using the glioma cell lines transfected with the control vector, sense MMP-2 and antisense MMP-2 would include a study on the regulation of MMP-2. Inducers of MMP-2 expression are generally thought to act at the level of transcriptional activation of the gene and promoter elements. Bian and Sun have shown MMP-2 to play a role in the regulation of the p53 promoter (Bian and Sun, 1997). Since LNZ-308 is p53 null, T98 has a p53 point mutation, and U87 contains wild-type p53, it would be interesting to compare the invasion of cell lines differing in p53 status. It has also been shown that irradiation might promote invasion-related gene expression by activation of wild-type p53 and a resulting increase of MMP-2 expression. Sublethal irradiation of rat glioma cells results in the formation of a greater number of tumor satellites in the rat brain *in vivo* connected with enhanced MMP-2 and reduced tissue inhibitor of metalloproteinases-2 expression (Wild-Bode, 2001). The newly created MMP-2 expression system can serve a valuable tool in future studies involving the affects of irradiation on p53, MMP-2 expression, and glioma invasion.

The *in vitro* invasion assays, as well as zymograms and Western blots, need to be repeated with and with out the addition of conA. In addition, the assays need to be conducted on the transfected T98 cells and the transfected LNZ-308 cells to see if similar data results from these different glioma cell lines. If a direct link between overexpression of MMP-2 and glioma invasion were seen in the *in vitro* invasion assays, the next step would be to conduct *in vivo* invasion assays using a rat glioma model for the human brain. The transfected cell lines could be fluorescently labeled to examine cellular invasion through the rat brain.

In conclusion, though these experiments do not conclusively demonstrate that MMP-2 plays the major role in glioma invasion, they do support the previous correlative studies and provide valuable tools for future work towards this goal.

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Biography

Nicole Danielle Sauers was born to Harry B. and Kathleen E. Sauers on December 1, 1977 in Philadelphia, Pennsylvania. She graduated from Mount Saint Joseph Academy in June of 1995. She attended LaSalle University where she earned a B.A. in Biology in May 1999 and minored in Spanish. She began graduate work in the Department of Biology at the University of Richmond in August of 1999. She completed her graduate work in June of 2001.